SABiosciences™

User Manual

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Methyl-Profiler™ DNA Methylation PCR Array System

Fast and Reliable Quantitative DNA Methylation Analysis without Bisulfite Conversion

See Purchaser Notification for limited use license and warranty information (page 3).

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SABiosciences Corporation 6951 Executive Way, Frederick, MD 21703 USA

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Fast and Reliable Quantitative DNA Methylation Analysis Without Bisulfite Conversion

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(For Catalog Numbers Prefixed by: MeA(H/M))

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I. Background and Introduction

Approximately 60 to 70% of all human gene promoters overlap with CpG islands, which are regions with an elevated GC content and a high frequency of CpG dinucleotides. Gene silencing by means of hyper-methylation of specific genes promoters is a well-known feature of neoplastic cells and also plays an important role in normal cell differentiation and development (1). DNA methylation occurs mainly at CpG dinucleotides and involves the enzymatic addition of a methyl group to the cytosine residue without changing the primary DNA sequences. Such modifications at regulatory regions (in particular gene promoters), correlate well with the transcriptional state of a gene: hyper-methylation represses transcription while hypo-methylation can lead to increased transcription levels. DNA methylation is an essential mechanism for normal cellular development, imprinting, X-chromosome inactivation, maintaining tissue specificity, and can contribute significantly to the progression of various human diseases.

The profiling of tumor suppressor genes and other key genes will allow the correlation of CpG island methylation status with transcriptional status, biological phenotypes or disease outcomes. Therefore, the results can provide insights into the molecular mechanisms and biological pathways critical for disease development and aid in the discovery and development of biomarkers.

DNA methylation detection technologies based on the bisulfite conversion of DNA samples, such as bisulfite sequencing and methylation specific PCR related methods, have greatly advanced the DNA methylation studies by providing scientists with the ability to analyze the methylome at single-base resolution. However, these technologies have several limitations that involve time constraints (bisulfite modification, amplification, cloning and sequencing), suboptimal DNA conversion yields, challenging primer design and PCR optimization, and low gene- and sample-throughput. In addition, most biological significant changes in DNA methylation are known to occur at multiple CpG dinucleotides simultaneously rather than at single bases, suggesting that a regional analysis can be representative for the methylation level of a CpG Island within a promoter region (gene) of interest (2).

The Methyl-Profiler DNA Methylation PCR System is an innovative technology enabling fast and accurate detection of CpG island DNA methylation profiles of individual genes as well as disease- or pathway-focused gene panels. This technology is an excellent alternative to low-throughput bisulfite-based methods. In brief, the method is based on the detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme (3). These enzymes will respectively digest unmethylated and methylated DNA. Following digestion, the remaining DNA is quantified by real-time PCR in each individual enzyme reaction using primers that flank a promoter (gene) region of interest. The relative fractions of (hyper) methylated, intermediate methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest. The reliability and simplicity of the procedure make this technology an ideal tool for semi-high throughput DNA methylation profiling and biomarker development for various research fields like stem cell differentiation and development, cancer and other human diseases.

Simple enzyme digestion + Real-Time PCR = Methyl-Profiler DNA methylation data highly comparable to bisulfite sequencing data.

References

1. Esteller M (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum Mol Genet* 6(1):R50-59.
2. Weber M*, et al.* (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 39(4):457-466.
3. Ordway JM*, et al.* (2006) Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets. *Carcinogenesis* 27(12):2409-2423.

1. Benefits

- **Fast reliable and quantitative: No bisulfite conversion. Ready to use.** Genomic DNA Enzyme Digestion → Real-Time PCR → Straightforward data analysis.
- **Disease or Pathway focused:** Simultaneously detect methylation status of 24 - 96 genes.
- **Genome Wide Coverage:** Pre-designed primers to detect the of methylation status of your gene of interest**.**

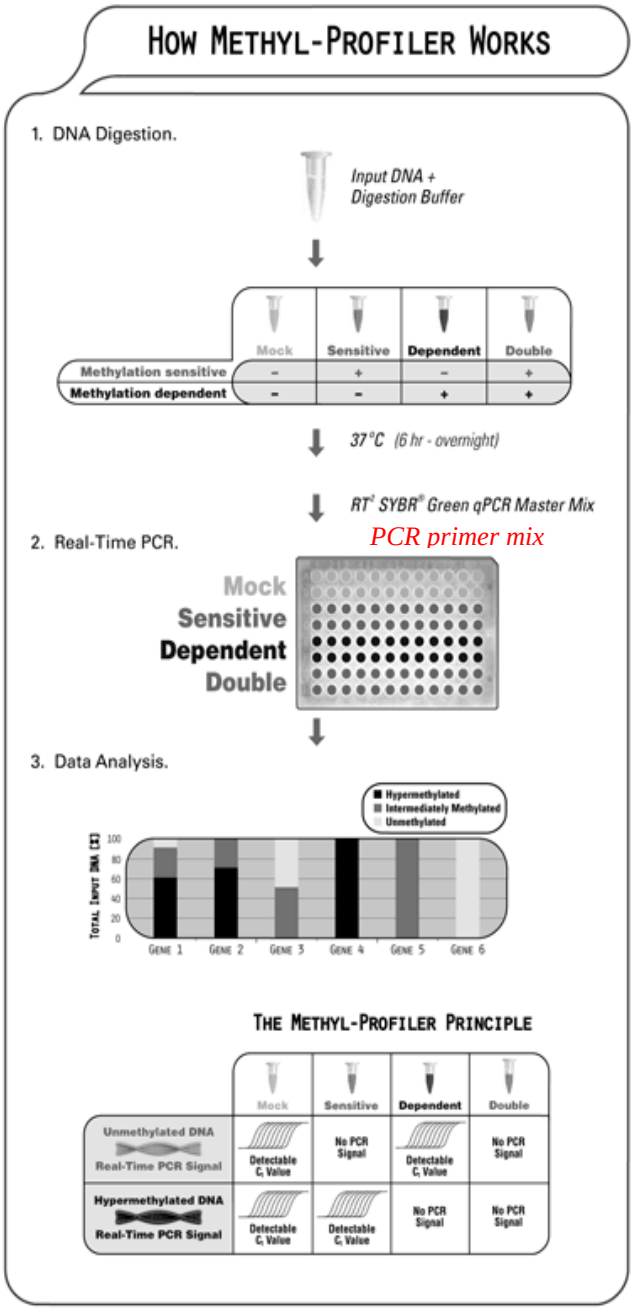


Figure 1: Methyl-Profiler DNA Methylation PCR Array Protocol Overview

1. DNA Digestion

Input genomic DNA is aliquoted into four equal portions:

a. Mock Digest (M_o)

No enzymes are added in this reaction. The product of the mock digestion represents the total amount of input DNA for real-time PCR detection.

b. Methylation Sensitive Digest (M_s)

Cleavage with a methylation-sensitive enzyme will digest unmethylated and partially methylated DNA. The remaining (hyper)-methylated DNA will be detected by real-time PCR.

c. Methylation Dependent Digest (M_d)

Cleavage with a methylation-dependent enzyme will preferentially digest methylated DNA. The remaining unmethylated DNA will be detected by real-time PCR.

d. Double Digest (M_{sd})

Both enzymes are added in the double digest, and all DNA molecules (both methylated and unmethylated) will be digested. This reaction measures the background and the fraction of input DNA refractory to enzyme digestion.

2. Real-Time PCR

Specific cycling conditions for many real-time PCR instruments are indicated in the manual.

3. Data Analysis

The relative amount of each DNA fraction (methylated, intermediate methylated and unmethylated) is calculated using a standard ΔC_t method, normalizing the amount of DNA in each digest against the total amount of input DNA in the M_o digest.

II. Materials Provided

The PCR arrays are available in five different plate formats, each tailored to a specific subset of real-time PCR instrument and associated blocks. Formats A, C, D, and F are 96-well plates, while Formats E and G are 384-well plates.

Format	For Real-Time Instrument	Plate
A	All ABI® standard blocks (7000, 7300, 7500, 7700, 7900) Bio-Rad® iCycler®, MyiQ™ Bio-Rad® Chromo 4 (MJ Research) Stratagene Mx3005p, Mx3000p	96-well
C	ABI® 7500 and 7900HT FAST 96-well blocks ABI® StepOne plus	96-well
D	Bio-Rad CFX96 Bio-Rad Opticon and Opticon 2 (MJ Research) Stratagene Mx4000	96-well
E	ABI® 7900HT (FAST) 384-well block	384-well
F	Roche LightCycler® 480, 96-well block	96-well
G	Roche LightCycler® 480, 384-well block	384-well

NOTE: *The format of a PCR Array is designated by the **last letter of the catalog number**. Before starting any experiment, **confirm** that you have the correct PCR Array format for your instrument.*

1. **Signature 24-Gene Panel, 96-Well PCR Arrays (MeA(H/M)-0#1A, C, D, and F)** are available and shipped in sets of two (2), twelve (12), or twenty-four (24).
2. **Signature 24-Gene Panel, 384-Well PCR Arrays (MeA(H/M)-0#1E, G)** are available and shipped in sets of four (4).
3. **Complete 96-Gene Panel, 96-Well PCR Arrays (MeA(H/M)-80#0A, C, D, F)** are available and shipped as a duplicate set of four different 24-gene, 96-well PCR Arrays.
4. **Complete 96-Gene Panel, 384-Well PCR Arrays (MeA(H/M)-30#0E, G)** are available and shipped in sets of two (2), twelve (12), or twenty-four (24).

Each PCR Array shipment includes the arrays and either twelve (12) optical thin-wall 8-cap strips (**Formats A & D**) or one (1) optical adhesive film (**Formats C, E, F & G**) per array.

Each 384-Well PCR Array format also includes one set of four 384-Well Plate EasyLoad Covers for each PCR Array provided in the package.

NOTE: *The PCR Arrays can only be used in 96-well and 384-well real-time PCR instruments. The PCR Arrays cannot be used in the Cepheid SmartCycler®, the Roche LightCycler® 2.0, or the Corbett Research Rotor-Gene™ and Rotor-Gene™ 6000.*

III. Additional Materials & Equipment Required

A. DNA Isolation Kit: See Recommendations in the Protocol Section.

B. DNA Methylation Enzyme Kit (MeA-03)
The MeA-03 kit contains all necessary components for the cleavage of methylated and unmethylated DNA and is **ESSENTIAL** for a **complete and successful** experiment. The reagents included in the kit are sufficient for processing 12 DNA samples.

C. RT² SYBR® Green qPCR Master Mixes:
These Master Mixes are **ESSENTIAL** for a **complete and successful** experiment. Be sure to select the correct formulation, size and quantity for your real-time PCR instrument.

Catalog #	Master Mix
PA-012	**RT² SYBR® Green / ROX qPCR Master Mix** Designed specifically for all ABI and Stratagene Instruments and Eppendorf mastercycler® ep *realplex* instruments with a ROX filter set.
PA-011	**RT² SYBR® Green / Fluorescein qPCR Master Mix** Designed specifically for BioRad iCylcer®, MyiQ™, and iQ™5 instruments.
PA-010	**RT² SYBR® Green qPCR Master Mix** Designed specifically for instruments that **do not** require a reference dye like: BioRad CFX96, BioRad Opticon™, Opticon™ 2 & Chromo™ 4 (MJResearch), Roche LightCycler® 480 System and Eppendorf mastercycler® ep *realplex* instruments without a ROX filter set.

For PCR Arrays and Master Mixes Combinations see below:

PCR Arrays		Master Mix	
Catalog #	**Pack Size**	**Catalog #**	**Quantity**
MeA(H/M)-0#1A, C, D, F	2	PA-01# 1	1
	12	PA-01#-12	1
	24	PA-01#-24	1
MeA(H/M)-0#1E, G	4	PA-01#-8	1
MeA(H/M)-80#0A, C, D, F	8	PA-01#	4
MeA(H/M)-30#0E, G	2	PA-01#	1
	12	PA-01#-12	1
	24	PA-01#-24	1

D. Additional Equipment and Reagents:

1. Real-Time PCR Instrument
2. Calibrated single- and multi-channel pipettes
3. RNase / DNase-free pipette tips and tubes
4. RNase / DNase-free 100-µL regular PCR reaction tubes (8- or 12-tube strings)
5. Molecular biology grade RNase- and DNase-free H_2O

IV. Protocol

***IMPORTANT*:** Please read through this entire protocol before beginning your experiment.

A. Tips for a Successful Assay

1. Avoiding DNA contamination

For reliable results, it is very important to prevent ***ANY*** contamination of the Methyl-Profiler Assay reactions with foreign DNA. Even very small amounts of foreign DNA can artificially inflate SYBR® Green signals, yielding false positive results. The most common source of contamination in the PCR reagents comes from the products of previous PCR experiments in your working area. Please follow the recommendations outlined below:

- Wear gloves throughout the entire procedure.
- Use only fresh PCR-grade reagents and lab ware.
- Physically separate your workspace for PCR setup and post-PCR work.
- Before setting up an experiment, decontaminate your PCR workspace and lab ware (pipette barrels, tube racks, etc.) with 10% bleach and UV light. Preferentially set up your reaction within a PCR workstation.
- Do not peel the protective film from the PCR Array until immediately before use.
- Close all tubes containing PCR products as soon as you finish transferring solutions.
- Treat any lab ware (tips or tubes) containing PCR products or other DNA with 10% bleach before discarding.

2. Genomic DNA Preparation and Quality Control

High quality DNA is ESSENTIAL for obtaining accurate results. The most important prerequisite for a successful DNA Methylation qPCR assay analysis is consistent, high-quality genomic DNA from every experimental sample. Therefore, sample handling and genomic DNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts or other contaminants will either degrade the DNA or decrease the restriction enzyme activities necessary for optimal DNA digestion.

- **Recommended Genomic DNA Preparation Method**
  The QIAGEN Blood & Tissue Kit is highly recommended for the preparation of genomic DNA samples. Ensure that samples have been treated for the removal of RNA, as RNA contamination will cause inaccuracies in DNA concentration measurements and can possibly affect restriction digestion efficiency. DO NOT omit the recommended RNase treatment step to remove RNA. If genomic DNA samples need to be harvested from biological samples where kits are not available, please contact Technical Support representatives for suggestions.

  For best results, resuspend or dilute all DNA samples in DNase-free water; or alternatively, in DNase-free 10 mM Tris buffer pH 8.0 without EDTA.

- **Measurement of DNA concentration and purity by UV spectrophotometry**
  Prepare dilutions of your genomic DNA samples and measure absorbance in DNase-free 10 mM Tris, pH 8.0 buffer. The spectral properties of nucleic acids are highly dependent on pH.

Recommended rations and values	
A260:A230	>1.7
A260:A280	> 1.8
A260 concentration	> 4µg/mL

- **DNA concentrations needed for restriction digestion and PCR assay**

  Using the recommended amount of DNA optimizes the sensitivity of detecting methylated DNA. More input DNA may be used if analyzing hypermethylated DNA isolated from samples of heterogeneous cell types, e.g. clinical tumor samples whereas heavy non-tumor cell contamination is expected, such as blood, or stromal cells etc. However, maintain the specific enzyme to DNA ratios outlined below for each assay, and purchase additional qPCR plates to ensure assay consistency.

3. Methyl-Profiler Enzyme Kit (MEA-03) Handling Guide

Important note:

- **DO NOT VORTEX ENZYMES**. Enzyme B is very sensitive to vortexing. Extensive vortexing can cause loss of enzyme activity. Instead, mix enzymes by gently pipetting up and down.

- Store enzymes at -20°C. When not at -20°C, enzymes should be kept on ice.

Catalog #	# Genes	Plate Format (# plates)	DNA Samples	gDNA starting material (µg)	gDNA per digestion reaction (µg)	gDNA per PCR assay (ng)
MeA(H/M)-0#1A,C,D,F	24	96-well (1)	1	1	~ 0.25	~ 10
MeA(H/M)-80#0A,C,D,F	96	96-well (4)	1	4	~ 1	~ 10
MeA(H/M)-30#0E,G	96	384-well (1)	1	2	~ 0.5	~ 4
MeA(H/M)-0#1E,G	24	384-well (1)	4	0.5*	~ 0.125*	~ 4

The table outlines the recommended genomic DNA input amounts associated with each Methyl-Profiler Assay format (columns 1-3). In addition, the amount of genomic DNA for each individual digestion (Mo, Ms, Md, Msd) reaction is listed, as well as the amount of genomic DNA PCR template that this translates to in the qPCR step. If more input DNA

per assay is desired, please order additional assays. *: amount for each of the four samples.

B. How to set up a Methyl-Profiler Assay

Four protocols are provided to assist you in setting up the restriction enzyme reactions and PCR assays. The setup is dependent on the PCR Array format used in the experiment.

Protocol	Catalog #	# Genes	Plate Format	DNA Samples	Page
B.1	MeA(H/M)-0#1A,C,D,F	24	96-well	1	12
B.2	MeA(H/M)-80#0A,C,D,F	96	96-well	1	15
B.3	MeA(H/M)-30#0E,G	96	384-well	1	17
B.4	MeA(H/M)-0#1E,G	24	384-well	4	20

B.1 Using a 24-genes 96-well PCR Array and one DNA sample.

Restriction digestion

B.1.1. Perform the restriction digestions with the Methyl-Profiler™ Enzyme Kit (Catalog # MeA-03).

B.1.2. Prepare a reaction cocktail without enzymes as indicated below. For this protocol it is recommended to use 1 µg genomic DNA. The 5X digestion buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer make sure to continue mixing the buffer until precipitates dissolve.

RNase/DNase-free H_2O	___ µl
5X Digestion Buffer	26 µl
Genomic DNA (1 µg)	___ µl
Final cocktail volume	120 µl

B.1.3. After adding H_2O to make the final cocktail volume 120 µl, vortex to thoroughly mix the components and spin down briefly.

B.1.4. Set up four digestion reactions (Mo, Ms, Md and Msd) according to the following table. ***All four tubes must contain equal amounts of genomic DNA.***

	Mo	Ms	Md	Msd
RNase/DNase-free H_2O	2 µl	1 µl	1 µl	
Cocktail from previous step	28 µl	28 µl	28 µl	28 µl
Enzyme A		1 µl		1 µl
Enzyme B			1 µl	1 µl
Total volume	30 µl	30 µl	30 µl	30 µl

B.1.5. Pipette up and down to thoroughly, yet gently, mix the components. Spin tubes briefly in microcentrifuge.

B.1.6. Incubate all four tubes at 37°C for 6 hours in a heating block or thermal cycler. The reaction can also be performed overnight.

B.1.7 After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 minutes.

B.1.8. The reactions are now ready for use or storage at -20°C. If samples are stored, please remember to vortex to thoroughly mix the samples after thawing. Spin down briefly before proceeding to the next step.

Setting up the PCR Reactions

B.1.9. Prepare individual PCR cocktails in a 1.5 mL tube for each of the four reactions (Mo, Ms, Md and Msd) from the previous step following the table below.

	Mo	Ms	Md	Msd
RNase/DNase-free H_2O	300 µl	300 µl	300 µl	300 µl
PCR Master Mix	330 µl	330 µl	330 µl	330 µl
Mo digest	30 µl			
Ms digest		30 µl		
Md digest			30 µl	
Msd digest				30 µl
Total volume	660 µl	660 µl	660 µl	660 µl

B.1.10. Mix tubes well by vortexing, and briefly spin down the solution to the bottom of the tube.

B.1.11. Carefully add 25 µl of the **Mo** cocktail to each well in **rows A & B** of the 96-well PCR Array, 25 µl of the **Ms** cocktail to each well in **rows C & D,** 25 µl of the **Md** cocktail to each well in rows **E & F**, and 25 µl of the **Msd** cocktail to each well in rows **G & H** as indicated below.

Digest	Well	1	2	3	4	5	6	7	8	9	10	11	12
M_o	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
M_s	C	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	D	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
M_d	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	F	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
M_{sd}	G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	H	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24

B.1.12. After loading the plate, carefully seal the plate or cap the wells. Centrifuge the plates briefly to remove air bubbles at 1000 rpm for 1 minute.

Running the PCR Reactions

B.1.13. To run the reactions, use the two-step program shown below for all cyclers. ***Note the unique cycling conditions necessary to ensure proper performance of the assay.***

Cycles	Temperature	Time
1	95°C	10 minutes[1]
40	97°C	15 seconds
	72°C	1 minute[2]
Melting curve segment	According to instrument recommendations	

[1]The 10 minutes step at 95°C is required to activate the HotStart DNA polymerase. [2]Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

B.1.14. After the run the data can be analyzed as described in section V.

B.2 Using a 96-genes 96-well PCR Array (4) and one DNA sample.

Restriction digestion

B.2.1. Perform the restriction digestions with the Methyl-Profiler™ Enzyme Kit (Catalog # MeA-03).

B.2.2. Prepare a reaction cocktail without enzymes as indicated below. For this protocol, it is recommended to use 4 µg genomic DNA. The 5X digestion buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer make sure to continue mixing the buffer until precipitates dissolves.

RNase/DNase-free H_2O	___ µl
5X Digestion Buffer	100 µl
Genomic DNA (4 µg)	___ µl
Final cocktail volume	470 µl

B.2.3. After adding H_2O to make the final cocktail volume 470 µl, vortex to thoroughly mix the components and spin down briefly.

B.2.4. Set up four digestion reactions (Mo, Ms, Md and Msd) according to the following table. ***All four tubes must contain equal amounts of genomic DNA.***

	Mo	Ms	Md	Msd
RNase/DNase-free H_2O	8 µl	4 µl	4 µl	
Cocktail from previous step	112 µl	112 µl	112 µl	112 µl
Enzyme A		4 µl		4 µl
Enzyme B			4 µl	4 µl
Total volume	120 µl	120 µl	120 µl	120 µl

B.2.5. Pipette up and down to thoroughly, yet gently, mix the components. Spin tubes briefly in microcentrifuge.

B.2.6. Incubate all four tubes at 37°C for 6 hours in a heating block or thermal cycler. The reaction can also be performed overnight.

B.2.7. After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 minutes.

B.2.8. The reactions are now ready for use or storage at -20°C. If samples are stored, please remember to thoroughly mix the samples after thawing. Spin down briefly before proceeding to the next step.

Setting up the PCR Reactions

B.2.9. Prepare individual PCR cocktails in a sterile 14 mL (Falcon) tube for each of the four reactions (Mo, Ms, Md and Msd) from the previous step following the table below.

	Mo	Ms	Md	Msd
RNase/DNase-free H_2O	1160 µl	1160 µl	1160 µl	1160 µl
PCR Master Mix	1280 µl	1280 µl	1280 µl	1280 µl
Mo digest	120 µl			
Ms digest		120 µl		
Md digest			120 µl	
Msd digest				120 µl
Total volume	2560 µl	2560 µl	2560 µl	2560 µl

B.2.10. Mix tubes well by vortexing, and briefly spin down the solution to the bottom of the tube.

B.2.11. Carefully add 25 µl of the **Mo** cocktail to each well in **rows A & B** of the four 96-well PCR Arrays; 25 µl of the **Ms** cocktail to each well in **rows C & D**; 25 µl of the **Md** cocktail to each well in rows **E & F**; and 25 µl of the **Msd** cocktail to each well in rows **G & H** as indicated below.

Digest	Well	1	2	3	4	5	6	7	8	9	10	11	12
M_o	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
M_s	C	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	D	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
M_d	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	F	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
M_{sd}	G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	H	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24

B.2.12. After loading each plate, carefully seal the plate or cap the wells. Centrifuge the plates briefly to remove air bubbles at 1000 rpm for 1 minute. One plate can be run immediately and the other three plates can be place at -20^{0}C until your PCR instrument is ready for another run. Do not thaw the plates before running the PCR but place them directly in the PCR instrument.

Running the PCR Reactions

B.2.13. To run the reactions, use the two-step program shown below for all cyclers. ***Note the unique cycling conditions necessary to ensure proper performance of the assay.***

Cycles	Temperature	Time
1	95°C	10 minutes[1]
40	97°C	15 seconds
	72°C	1 minute[2]
Melting curve segment	According to instrument recommendations	

[1]The 10-minute step at 95°C is required to activate the HotStart DNA polymerase. [2]Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

B.2.14. After the run the data can be analyzed as described in section V.

B.3 Using a 96-genes 384-well PCR Array and one DNA sample.

Restriction digestion

B.3.1. Perform the restriction digestions with the Methyl-Profiler™ Enzyme Kit (Catalog # MeA-03).

B.3.2. Prepare a reaction cocktail without enzymes as indicated below. For this protocol, it is recommended to use 2 µg genomic DNA. The 5X digestion buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer make sure to continue mixing the buffer until precipitates dissolves.

RNase/DNase-free H_2O	___ µl
5X Digestion Buffer	100 µl
Genomic DNA (2 µg)	___ µl
Final cocktail volume	470 µl

B.3.3. After adding H_2O to make the final cocktail volume 470 µl, vortex to thoroughly mix the components and spin down briefly.

B.3.4. Set up four digestion reactions (Mo, Ms, Md and Msd) according to the following table. ***All four tubes must contain equal amounts of genomic DNA.***

	Mo	**Ms**	**Md**	**Msd**
RNase/DNase-free H_2O	4 µl	2 µl	2 µl	
Cocktail from previous step	116 µl	116 µl	116 µl	116 µl
Enzyme A		2 µl		2 µl
Enzyme B			2 µl	2 µl
Total volume	120 µl	120 µl	120 µl	120 µl

B.3.5. Pipette up and down to thoroughly, yet gently, mix the components. Spin tubes briefly in microcentrifuge.

B.3.6. Incubate all four tubes at 37°C for 6 hours in a heating block or thermal cycler. The reaction can also be performed overnight.

B.3.7. After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 minutes.

B.3.8. The reactions are now ready for use or storage at -20°C. If samples are stored, please remember to thoroughly mix the samples after thawing. Spin down briefly before proceeding to the next step.

Setting up the PCR Reactions

B.3.9. Prepare individual PCR cocktails in a 1.5 mL tube for each of the four reactions (Mo, Ms, Md and Msd) from the previous step following the table below.

	Mo	**Ms**	**Md**	**Msd**
RNase/DNase-free H_2O	470 µl	470 µl	470 µl	470 µl
PCR Master Mix	590 µl	590 µl	590 µl	590 µl
Mo digest	120 µl			
Ms digest		120 µl		
Md digest			120 µl	
Msd digest				120 µl
Total volume	1180 µl	1180 µl	1180 µl	1180 µl

B.3.10. Mix tubes well by vortexing, and briefly spin down the solution to the bottom of the tube.

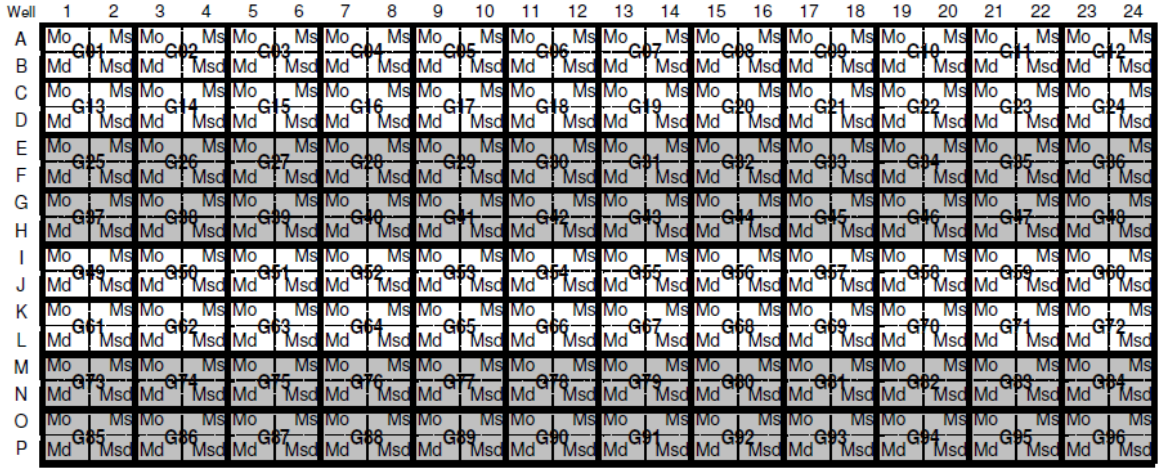
B.3.11. Carefully add each cocktail to the appropriate wells of the 384-well plate, using the provided 384-Well Plate **Easy Load** Covers as follows:

Place **Cover #1** on the plate. Add 10 µL of **Mo** cocktail to the open wells (Odd number wells of rows A, C, E, G, I, K, M and O). Remove & discard the cover.

Place **Cover #2** on the plate. Add 10 µL of **Ms** cocktail to the open wells (Even number wells of rows A, C, E, G, I, K, M and O). Remove & discard the cover.

Place **Cover #3** on the plate. Add 10 µL of **Md** cocktail to the open wells (Odd number wells of rows B, D, F, H, J, L, N and P). Remove & discard the cover.

Place **Cover #4** on the plate. Add 10 µL of **Msd** cocktail to the open wells (Even number wells of rows B, D, F, H, J, L, N and P). Remove & discard the cover.



B.3.12. After loading the plate, carefully seal the plate or cap the wells. Centrifuge the plate briefly to remove air bubbles at 1000 rpm for 1 minute.

Running the PCR Reactions

B.3.13. To run the reactions, use the two-step program shown below for all cyclers. ***Note the unique cycling conditions necessary to ensure proper performance of the assay.***

Cycles	Temperature	Time
1	95°C	10 minutes[1]
40	97°C	15 seconds
	72°C	1 minute[2]
Melting curve segment	According to instrument recommendations	

[1]The 10 minute step at 95°C is required to activate the HotStart DNA polymerase. [2]Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

B.3.14. After the run the data can be analyzed as described in section V.

B.4 Using a 24-genes 384-well PCR Array and four DNA samples.

Restriction digestion

B.4.1. Perform the restriction digestions with the Methyl-Profiler™ Enzyme Kit (Catalog # MeA-03).

B.4.2. Prepare a reaction cocktail without enzymes as indicated below. For this protocol it is recommended to use **0.5 µg genomic DNA *per sample***. The 5X digestion buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer make sure to continue mixing the buffer until precipitates dissolves.

RNase/DNase-free H_2O	___ µl
5X Digestion Buffer	27 µl
Genomic DNA (0.5 µg)	___ µl
Final cocktail volume	125 µl

B.4.3. After adding H_2O to make the final cocktail volume 125 µl, vortex to thoroughly mix the components and spin down all four tubes briefly.

B.4.4. Set up four digestion reactions (Mo, Ms, Md and Msd) for each DNA sample according to the following table. If all done at once you will have 4 digestion tubes for each DNA sample (a total of 16 tubes). ***All four digestion tubes must contain equal amounts of genomic DNA.***

	Mo	**Ms**	**Md**	**Msd**
RNase/DNase-free H_2O	1 µl	0.5 µl	0.5 µl	
Cocktail from previous step	29 µl	29 µl	29 µl	29 µl
Enzyme A		0.5 µl		0.5 µl
Enzyme B			0.5 µl	0.5 µl
Total volume	30 µl	30 µl	30 µl	30 µl

B.4.5. Pipette up and down to thoroughly, yet gently, mix the components. Spin tubes briefly in microcentrifuge.

B.4.6. Incubate all four tubes at 37°C for 6 hours in a heating block or thermal cycler. The reaction can also be performed overnight.

B.4.7. After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 minutes.

B.4.8. The reactions are now ready for use or storage at -20°C. If samples are stored, please remember to thoroughly mix the samples after thawing. Spin down briefly before proceeding to the next step.

Setting up the PCR Reactions

B.4.9. Prepare individual PCR cocktails in a 1.5 mL tube for each of the four reactions (Mo, Ms, Md and Msd) from the previous step following the table below. Alternatively the H_2O and PCR Master Mix can be mixed together and then carefully aliquoted into the required amount of tubes (for 4 samples this will be 16 tubes) where after the digestion mixtures from the previous step can be added.

	Mo	**Ms**	**Md**	**Msd**
RNase/DNase-free H_2O	140 µl	140 µl	140 µl	140 µl
PCR Master Mix	170 µl	170 µl	170 µl	170 µl
Mo digest	30 µl			
Ms digest		30 µl		
Md digest			30 µl	
Msd digest				30 µl
Total volume	340 µl	340 µl	340 µl	340 µl

B.4.10. Mix tubes well by vortexing, and briefly spin down the solution to the bottom of the tube.

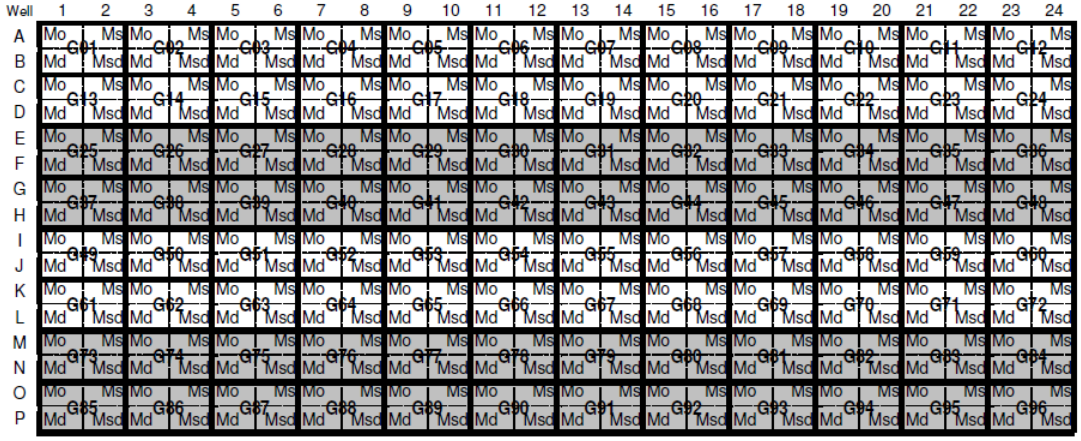
B.4.11. Carefully add each cocktail to the appropriate wells of the 384-well plate using the provided 384-Well Plate **Easy Load** Covers as follows:

Place **Cover #1** on the plate. Add 10 µL Mo cocktail to the open odd numbered wells of rows A & C for **Sample 1**, E & G for **Sample 2**, I & K for **Sample 3**, and M & O for **Sample 4**. Remove & discard the cover.

Place **Cover #2** on the plate. Add 10 µL Ms cocktail to the open even numbered wells of rows A & C for **Sample 1**, E & G for **Sample 2**, I & K for **Sample 3**, and M & O for **Sample 4**. Remove & discard the cover.

Place **Cover #3** on the plate. Add 10 µL Md cocktail to the open odd numbered wells of rows B & D **for Sample 1**, F & H for **Sample 2**, J & L for **Sample 3**, and N & P for **Sample 4**. Remove & discard the cover.

Place **Cover #4** on the plate. Add 10 µL Msd cocktail to the open even numbered wells of rows B & D for **Sample 1**, F & H for **Sample 2**, J & L for **Sample 3**, and N & P for **Sample 4**. Remove & discard the cover.



B.4.12. After loading the plate, carefully seal the plate or cap the wells. Centrifuge the plate briefly to remove air bubbles at 1000 rpm for 1 minute.

Running the PCR Reactions

B.4.13. To run the reactions, use the two-step program shown below for all cyclers. ***Note the unique cycling conditions necessary to ensure proper performance of the assay.***

Cycles	Temperature	Time
1	95°C	10 minutes[1]
40	97°C	15 seconds
	72°C	1 minute[2]
Melting curve segment	According to instrument recommendations	

[1]The 10 minutes step at 95°C is required to activate the HotStart DNA polymerase. [2]Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

B.4.14. After the run the data can be analyzed as described in section V.

V. Data analysis

1. Obtaining the raw Threshold Cycle (C_t) Values

After the cycling program is completed, obtain the C_t values following the instructions provided by the manufacturer of your Real-Time PCR Instrument. We recommend manually setting the Baseline and Threshold Values according to the following directions. Note that if you would like to compare multiple plates make sure that **the settings for all plates are identical.**

a. **Baseline:** Using the Linear View of the amplification plots, set the instrument to use the readings from cycle number 2 through the cycle just before the earliest visible amplification, usually between cycle 10 and 15.
b. **Threshold Value:** Using the Log View of the amplification plots, place the threshold above the background signal but within the lower third of the linear portion of the amplification curves.

2. Exporting C_t Values

Export and/or copy-and-paste the C_t values from your instrument software to a blank Excel spreadsheet following the instructions provided by your instrument manufacturer.

3. Excel-Based Data Analysis Template

a. Download the Methyl-Profiler PCR Array Excel-based data analysis template that matches the gene panel and plate format that you used from our website at:
http://www.sabiosciences.com/methylationdataanalysis.php
Or click the "DNA Methylation Data Analysis" link found in the lower "Product Support" section of the gray right-hand sidebar of any DNA Methylation web page.

b. Paste in your C_t value data and analyze the automatically generated results by simply following the directions in the "Instructions" worksheet of the Excel file.

3. Data Quality Control

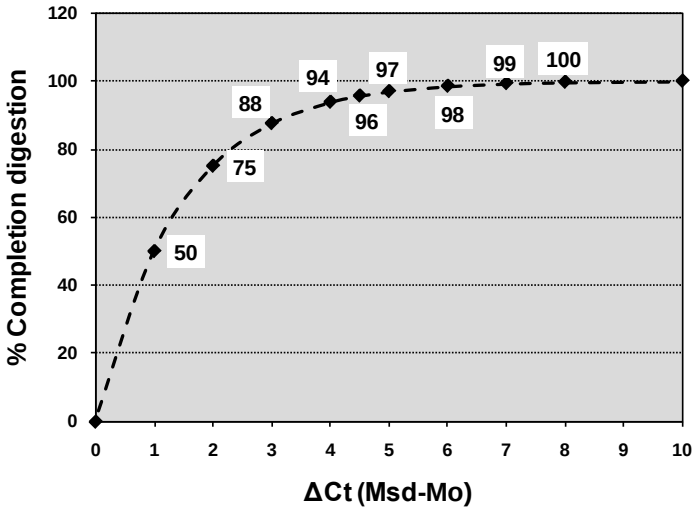
a. **Mock Digest (M_o) C_t Values**
The C_t values of the mock digests for all genes should be within a range of 21 to 29 cycles if the recommended amounts of genomic DNA were used.

b. **Single Enzyme Digest (M_s and M_d) C_t Values**

The C_t values of the M_s and M_d digests should be between the values of the mock and double digests, depending on the methylation status of the DNA samples.

c. **Double Digest (M_{sd}) C_t Values**
The C_t values of the double digests should be higher than the C_t values for the mock digest or single digests for the same gene.

d. **Enzyme Digestion Efficiency**
The difference in C_t values between the double and mock digests should be greater than two [ΔCt ($M_{sd} - M_o$) > 2] and represent the analytical window (W) of the assay. When W is greater than 2, it means that more that 75% of all DNA molecules in the samples were digested; hence the results are reliable and meaningful. See also the "Data QC Report" worksheet in the Excel data analysis template. For each and every gene, the analytical window (W) values should be greater than 2 and the R values should be less than 25%.



e. **Dissociation (Melting) Curve**
Perform the default melting curve program on your instrument immediately after the cycling program. Generate the first derivative dissociation curve for each well in each plate using your instrument's software. A single well define peak should appear in each well. If your instrument does not have a default melting curve program, run following program instead:

95°C, 1 min; 65°C, 2 min (OPTICS OFF)
65°C to 95°C at 2 °C / sec (OPTICS ON)

4. Data Analysis and Interpretation

a. **Relative Amounts of DNA in each Methylated Fraction**
The "Results" worksheet displays the relative percentage of (hyper)-methylated (HM), intermediately methylated (IM) and unmethylated (UM) DNA in each target genomic DNA sequence. The HM values can be used to generate a graphical

representation of the data using our developed Hierarchical Clustering method (**http://www.sabiosciences.com/dna_methylation_data_analysis.php**).

b. **Significance of Methylation Results**

The level of HM methylation considered to be significant (potential positive marker for hyper-methylation) must be defined by the researcher. However, the same principles used to define the significance of bisulfite-based sequencing and real-time PCR based methods apply to the Methyl-Profiler™ PCR Arrays and qPCR Assays as well. Alternatively, to define whether your results are significant you might take the following into consideration.

- *Percent of (Hyper)-methylated DNA.* In most cases, only hyper-methylated promoters will repress gene expression. Therefore, the minimum level of hyper-methylation considered to be positive can be set at 10 to 20% (similar as defined for bisulfite-based PCR methods). However, this is dependent on the ratio target vs. non-target cells present in the sample (i.e. normal cells mixed with cancerous cells). The greater the extent of contamination, the higher the threshold must be set.
- *Comparison between a Control and Experimental DNA Samples***.** Such parallel analysis will allow you to see if the methylation status of an experimental sample is substantially different from a matched control sample (i.e. tumor sample vs. normal control or treated sample vs. untreated).
- *Low (Hyper) methylation (< 10%) and High Intermediate Methylation (> 60%).* Intermediate methylated DNA may have biological significance if such methylation status is associated with a specific tumor, tissue, or other phenotype. Ideally, to determine if this methylation status is sufficient to repress transcription, one should consider measuring the corresponding expression levels and compare those with the expression levels in the appropriate controls.

5. The ΔC_t Data Analysis Method

The advantage of using Real-Time PCR for the detection of remaining target DNA copies is the use of the cycle threshold (C_t) values which are inversely proportional to the amount of target DNA in the sample. Also the product amount doubling after each cycle allows the total input ($\mathbf{C_{Mo}}$) to be defined as

$$\mathbf{C_{Mo} \sim 2^{-CtMo}}$$

A specific data analysis excel template was developed in order to calculate the relative amount of methylated DNA in each of the four assays using the ΔCt method. The amount of remaining DNA in each digest is normalized to the total amount of input DNA (mock digest). Therefore the relative amount of target DNA copies that are resistant to enzyme digestion ($\mathbf{C_R}$) are defined as: $\mathbf{C_R = 2^{-(CtMsd-CtMo)} = 2^{-\Delta Ct\ (Msd-Mo)}}$; in which **Ct Msd** represents the Ct values for the double digest assay. This amount is subtracted as background signal in the calculations.

The amount of hyper methylated target DNA copies (C_{HM}) is defined as: $C_{HM} = (2^{-CtMs} - C_R) / (2^{-CtMo} (1 - C_R)) = (2^{-\Delta Ct\ (Ms-Mo)} - C_R) / (1 - C_R)$; in which **Ct Ms** represent the Ct values for the MSRE digest.

The amount of un-methylated DNA (C_{UM}) can be determined as: $(2^{-CtMd} - C_R) / (2^{-CtMo} (1 - C_R)) = (2^{-\Delta Ct\ (Md-Mo)} - C_R) / (1 - C_R)$, in which **Ct Md** represent the Ct values for the MDRE digest.

If the sum of C_{HM} and C_{UM} is smaller than 100, the amount of intermediately methylated DNA (C_{IM}) equals ($1-C_{UM} - C_{HM}$).

The Ct values can vary up to 1.0 cycle dependent on technical errors and well to well variations within Real-Time PCR instruments. In order to eliminated some of these variations it is recommended to use larger ΔCt numbers for the calculations of the relative amount of methylated and hyper methylated target DNA copies. In our excel template when the ΔCt values are smaller than 1.0 the formulas are modified as indicated below:

ΔCt (Ms-Mo) < 1.0 and ΔCt (Md-Mo) ≥ 1.0 then $C_{HM} = 1 - C_{UM}$

ΔCt (Md-Mo) < 1.0 and ΔCt (Ms-Mo) ≥ 1.0 then $C_{UM} = 1 - C_{HM}$

ΔCt (Md-Mo) < 1.0 and ΔCt (Ms-Mo) < 1.0 then $C_{UM} = C_{HM}$ = 50% methylation

VI. Troubleshooting and FAQs

A. Troubleshooting Guide

	Comments and suggestions
1. Incomplete Restriction Enzyme Digestion: C_t (M_{sd}) –C_t (M_o) < 2	
Incomplete digestion dramatically reduces the sensitivity of the Methyl-Profiler™ PCR Array. Complete digestion is guaranteed when using the Methyl-Profiler™ DNA Methylation Enzyme Kit when the instructions are followed carefully. The most common reason for incomplete digestion is poor DNA sample quality. Common reasons for incomplete digestion are:	
a) Low Restriction Enzyme Activity	Be sure that the Methyl-Profiler™ DNA Methylation Enzyme Kit has not expired. Be sure to use the correct amount of both enzymes recommended in this protocol for the DNA amount used.
b) RNA Contamination in the DNA Samples	RNA contamination inhibits restriction enzyme DNA digestion while also causing an overestimation of the DNA concentration. Be sure to include any RNase treatment steps recommended in the procedure of your chosen DNA isolation kit.
c) Other Contaminants in the DNA Samples	DNA prepared from difficult organ tissues may contain protein and/or polysaccharide contaminants that significantly inhibit restriction enzyme activity. Organic reagents (such as chloroform, phenol, and isopropanol) used in some DNA kits and protocols may not be completely removed. Be sure to use the recommended DNA isolation kits and protocols and avoid using organic solvent-based methods and protocols for DNA isolation.
d) Too Much DNA Used in the Digestion	Be sure to use at least a four-hour incubation time at 37°C as well as the tubes with recommended size in the protocol. Switch to an overnight incubation if a shorter time was previously used and caused incomplete digestion.
e) Incorrect Incubation Conditions	Be sure to use at least a six-hour incubation time at 37°C as well as the tubes with recommended size in the protocol. Switch to an overnight incubation if a shorter time was previously used and caused incomplete digestion

Comments and suggestions

2. High Mock Digestion (M_o) C_t Values from Most if Not All Genes

a) Not Enough DNA Used in the Digestion	Be sure to use at least the amount of DNA recommended in this protocol and to use the recommended methods and instruments to determine DNA concentrations. Also, be sure to include any RNase treatment steps recommended in the procedure of your chosen DNA isolation kit.
b) Degraded DNA	DNA samples are contaminated by microbes due to improper storage of your DNA samples such as at 4 °C. Always store your DNA samples at -20 °C (more than 2 years), and at -80 °C (indefinitely).
c) Improper PCR Array or Master Mix Storage	Storing these components at inappropriate temperature for extended period reduces their activity and PCR amplification efficiency.
d) Incorrect Real-Time PCR Cycling Program Used	Be sure that you used the correct cycling program that includes 10 minutes at 95°C, which is required to fully activate the Hot Start enzyme in the RT^2 SYBR® Green qPCR Master Mix.

3. All Four Digests' (M_o, M_s, M_d, and M_{sd}) C_t Values for an Individual Gene $\geq$ 32

Your DNA sample may contain a different sequence relative to the most recent NCBI Genome Build due to unreported chromosomal abnormalities (insertion or deletions) or single nucleotide polymorphisms (SNPs) that affect the Methyl-Profiler PCR Assays. Verification of this situation may require sequencing of the relevant genomic region in your original DNA sample. Another reason is:

a) Homozygous Deletions	If the C_t values from all the four digests for an individual gene, but not the majority of genes, are equal to or greater than 32, genomic homozygous deletion most likely exists at this locus in the genomic DNA of your original sample.

B. Frequently Asked Questions

	Comments
1. How can I be sure that the restriction enzyme digestion is complete?	An excess of each restriction enzyme in combination with a long incubation time assures a complete digestion when the recommended DNA amounts are used. The larger the difference between the C_t values of the mock and double digests [$W = \Delta C_t\ (M_o) - C_t\ (M_{sd})$], the more complete the restriction enzyme digestion. Because C_t values are inversely and exponentially related to the initial amount of DNA material, each unit of cycle difference between these digests represents an additional two-fold difference in the amount of DNA. For example, a five-cycle difference means that the double digest contains only (100 x 2 ^ -C_t = 100 x 2 ^ 5 =) 3.125 percent of the DNA of the mock digest, meaning that the reaction is therefore (100 - 3.125 =) 96.875 percent complete.
2. Can this technology reliably characterize heterogenous tissue samples?	Thanks to the sensitive and quantitative nature of real-time PCR, hyper-methylated DNA can be detected even when the relevant cells are present at only five percent of a total population. This level of sensitivity equals bisulfite-sequencing and bisulfite-PCR methods. In contrast, Methylation-Specific PCR (MSP) only detects sequences when bisulfite conversion is 100 percent successful, thereby having decreasing the assay sensitivity.
3. How are the primers in the DNA methylation PCR Array designed?	The primers are designed around CpG islands known to be hyper-methylated under relevant biological conditions or in relevant biological samples. Besides the usual specific requirements that real-time PCR primers must meet, the amplicon sequence must contain restriction sites for both the methylation-sensitive and methylation-dependent enzymes. As a result, the amplicon lengths are around 150 to 400 bp but do not affect the PCR efficiency. The design algorithm also accounts for the GC-rich nature of genomic DNA sequences that tend to make primer design more difficult, especially in and around CpG islands. Each primer pair is also experimentally validated for a single dissociation curve peak, and consistently high amplification efficiencies

	Comments
4. Do I need an internal methylated DNA reference?	No. For any given target sequence, the same primer pair is used to amplify the four different digests allowing a direct and reliable comparison. In contrast, bisulfite modification-based PCR methods use two different pairs of primers to amplify either methylated or unmethylated templates of a given target sequence after conversion. Differences in their amplification efficiencies cause biased results requiring normalization to *in vitro* methylated reference DNA. However, its primers must also have similar amplification efficiencies and its methylation must be consistently complete, ideals only achieved after careful trial and error based optimization and additional cost.
5. Does this method detect methylation at specific CpG dinucleotides?	No. This method examines the methylation status across a CpG-rich sequence. Other methods only analyze one or two CpG sites in one assay and assume that their methylation status represents the status of the surrounding target sequence. These methods include Southern blot, MSRE and RLGS, and bisulfite conversion-based methods like MSP, TaqMan-based MethyLight, MS-SNuPE, GoldenGate DNA Methylation BeadArray, Infinium DNA Methylation BeadChip, and COBRA.
6. Can I reliably detect intermediate methylated DNA with this technology?	Yes. Hyper-methylated (> 60 percent methylated), and unmethylated (0 percent methylated) amounts of DNA in a sample are each directly and reliably detected. The total amount of input DNA minus the hyper-methylated and unmethylated DNA fractions yields the amount of intermediate methylated DNA (between 0 and 60 percent methylated). Lower extents of hyper-methylation have been more and more correlated with biological phenotypes when concurrent with larger extents of intermediate methylation levels.
7. Will pipetting error affect the Methyl-Profiler™ PCR Array results?	The passive reference dyes in the PCR master mixes, such as ROX and Fluorescein, are used by the real-time PCR instruments to normalize variation from well to well. Therefore, these systems tolerate volume variations caused by pipetting error or evaporation. Even a 20% pipetting error causes only 0.05-cycle differences in C_t values.
8. How can I prevent the evaporation of reaction volume from the wells?	Carefully and completely seal the PCR Array with the optical thin-wall 8-cap strips or the optical adhesive film before placing it into your thermal cycler.

If you have additional questions or technical difficulties, please check our website (www.sabiosciences.com) for a more complete listing of Frequently Asked Questions (FAQs), or call our Technical Support Representatives at 1-888-503-3187 or 301-682-9200.

NOTES

NOTES

Methyl-Profiler™ DNA Methylation PCR Array User Manual

Part #1038A Version 2.1 3/10/2010

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